

The patient was enrolled in a gene therapy trial based on autologous gene-corrected hematopoietic stem cells ([clinicaltrials.gov](http://clinicaltrials.gov) #NCT01515462), mobilized with G-CSF and plerixafor. Treatment with anakinra was discontinued 48 hours before mobilization, but was soon restarted because of the increase in white blood cells and inflammation indexes with exacerbation of skin lesions, arthralgia, and hematuria, and led again to a rapid laboratory and clinical remission (data not shown). Notably, the use of anakinra allowed a successful mobilization with G-CSF without the occurrence of other autoinflammatory manifestations. To our knowledge, this is the first reported case of use of IL-1R blocker in a patient with WAS, with clinical benefit.

This case is very emblematic for several reasons. Whole-genome sequencing complemented by specific breakpoint sequencing allowed the identification of the inversion with intact exon sequences, elucidating the previous normal genetic analysis. Complex genomic rearrangements involving inversions are generally noncanonical gene conversion events<sup>6</sup> and could have occurred in an ancestor allele in the family through a *de novo* mutation occurring in the mother.

Autoimmune and autoinflammatory manifestations in patients with WAS typically present early in life, are often refractory to therapy, and are associated with a worse clinical prognosis and an increased risk of developing a malignancy.<sup>3,7</sup> Our patient's autoinflammatory manifestations were resistant to several immunosuppressive drugs and the use of CyA was associated with a severe viral complication. Anakinra dramatically improved PG, vasculitis, and arthritis, showed a good safety profile, and allowed stabilization of the patient for definitive treatment. The response to anakinra suggests that the dysregulation of the innate immune system is involved in the genesis of autoinflammatory manifestations in patients with WAS and shows that IL-1 may serve in selected cases as a target for therapy, avoiding the use of other classes of immunosuppressors that can increase the risk for severe infections.

It has been hypothesized that defects in chemotaxis and podosomes formation in WASp-deficient cells may favor the onset of autoinflammatory manifestations. In addition, a recent study in a patient with aggressive PG showed a critical role for proline-serine-threonine phosphatase interacting protein 1, which is involved in cytoskeletal regulatory functions through interaction with WASp, in the Pyogenic Arthritis, Pyoderma gangrenosum, and Acne syndrome.<sup>8</sup> A greater understanding of the role of WASp in inflammation and of potential pathways that may be targeted therapeutically to modulate immunity in WAS is desirable to improve the management of the affected patients while waiting for definitive treatment by stem cell transplantation or gene therapy.

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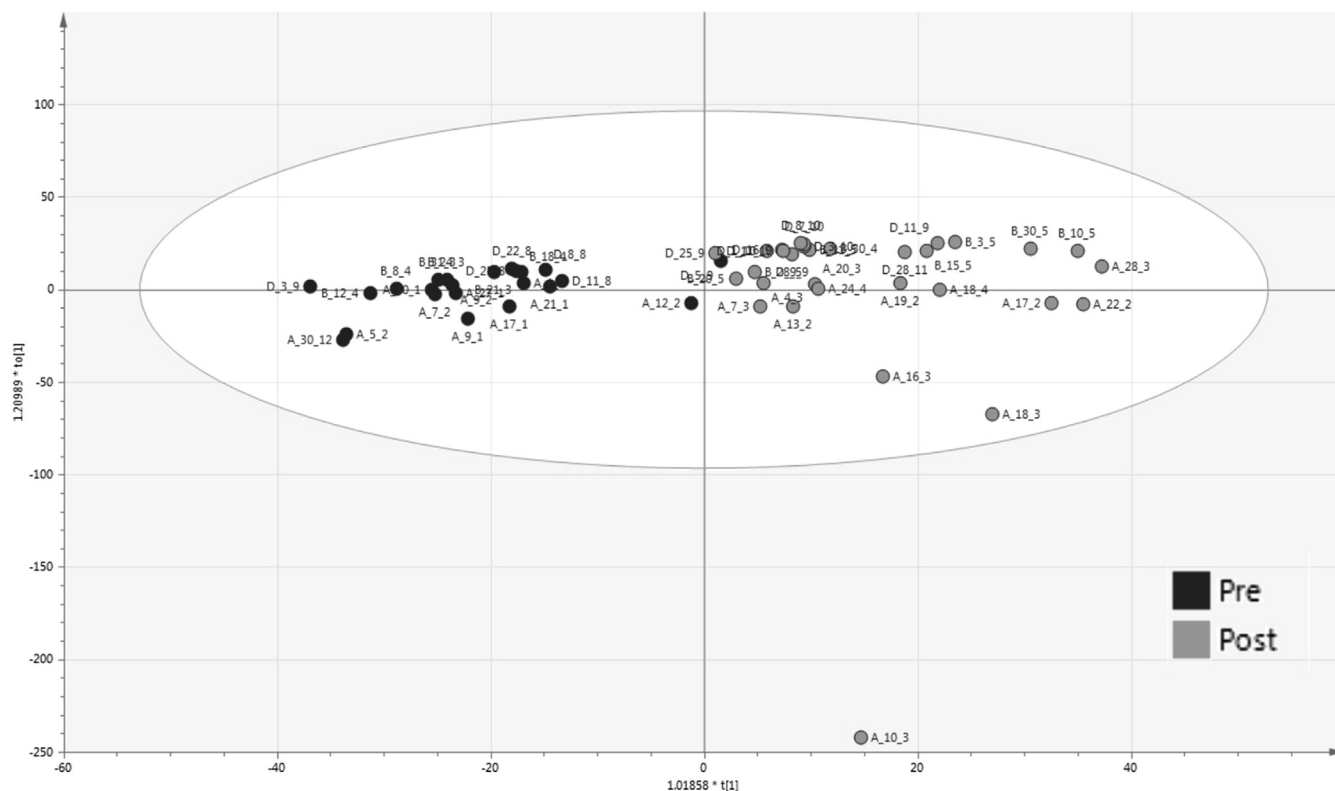
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## Functional changes in gut microbiota during hematopoietic stem cell transplantation for severe combined immunodeficiency



### To the Editor:

Severe combined immunodeficiencies (SCIDs), a group of rare, inherited conditions characterized by T lymphocytopenia, are fatal before age 1 year without hematopoietic stem cell transplantation (HSCT). Graft-versus-host disease (GvHD) and bacteremia cause post-HSCT morbidity and mortality. Early studies showed that germ-free mice fail to develop GvHD, thus demonstrating a role for microbiota in GvHD pathogenesis.<sup>1</sup> In



**FIG 1.** PLS-DA showing separation of metabolite profiles by pre- or post-HSCT status. X/Y overview values for pre- and post-HSCT:  $R^2 = 0.77$ ,  $Q^2 = 0.6$ ; values  $>0.5$  demonstrate data passes validation and are robust and reproducibly predictive of pre- and post-HSCT grouping.

adult HSCT, low gut microbiota diversity and bacterial dominance are associated with development of gut GvHD<sup>2</sup> and bacteremia<sup>3</sup> and low microbial diversity is predictive of transplant-related mortality.<sup>4</sup> We previously demonstrated that bacterial taxonomy of the gut microbiota in 3 patients with SCID changed over time, producing distinct pre- and post-HSCT microbiota populations with low microbial diversity and bacterial dominance.<sup>5</sup> Recently, research has focused on microbiota function in addition to taxonomy because microbial products can have immunoregulatory roles.

We studied changes in gut microbiota functional profiles through HSCT for 3 patients with SCID who underwent a transplant who have had their microbiota structure previously published.<sup>5</sup> Clinical data and daily stool samples were collected following informed parental consent. Stool samples were stored at  $-80^{\circ}\text{C}$  before analysis. Disease, demographic characteristics, and HSCT details for the 3 patients are reproduced from previously published data (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).<sup>5</sup> Details of computational prediction of microbiota functions and metabolic profiling are provided (see this article's Methods section in the Online Repository at [www.jacionline.org](http://www.jacionline.org)).

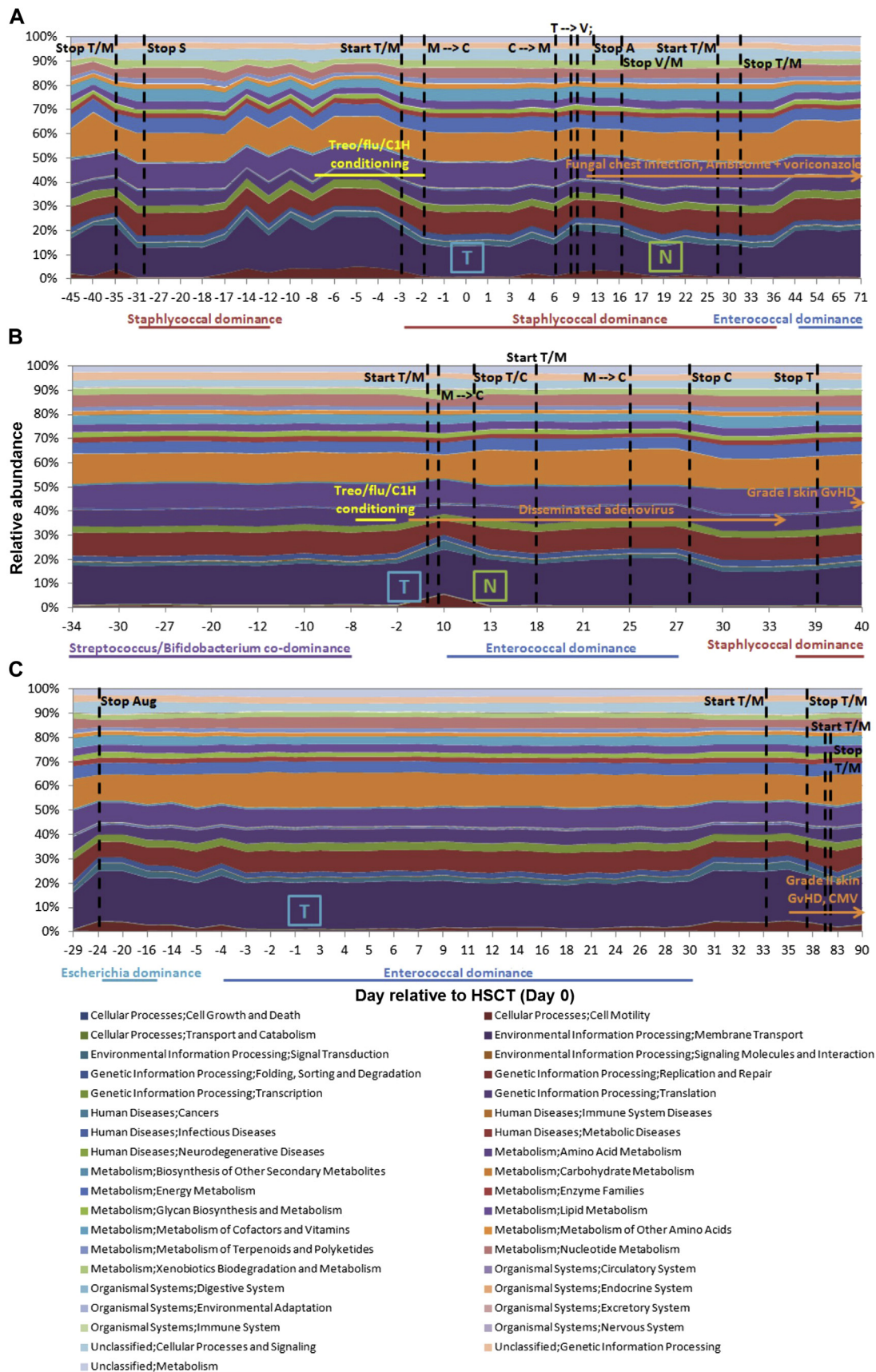
Metabolomic profiles obtained from ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) of 54 stool samples spanning pre- and post-HSCT were analyzed using partial least squares discriminant analysis (PLS-DA; Fig 1). Combining all patients in one analysis shows that samples can be separated into pre- and post-HSCT groups. The pre-HSCT samples most closely related to the post-HSCT group were collected in the few days preceding HSCT. Masses and retention

times of 5454 metabolites found across the 54 samples are provided (see UPLC-MS Data file in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Coefficient analysis demonstrates that 280 metabolites were significantly associated with pre-HSCT status and 1060 with post-HSCT status. Because of the large number of associated metabolites and limitations in metabolomics analysis, further metabolite identification was not performed.

The results of the PLS-DA analysis of the gut metabolome based on UPLC-MS/MS are similar to results for microbiota structure<sup>5</sup>: samples can be separated into pre- and post-HSCT groups and reveal a gradual shift from a pre- to post-HSCT metabolome, suggesting that HSCT does not cause sudden profound shifts in microbial function. The implications of these shifts for patients with SCID are not yet clear. The changes seem to be influenced by new therapeutic strategies such as probiotics. However, unlike microbiome analysis, identification of metabolites based on neutral mass is troublesome because of underdeveloped databases of stool metabolites. Over time these limitations will resolve, improving annotation of significant metabolites. Nonetheless, *profiling* these functional small molecules offers important insights into host and microbe activity through transplant.

The PLS-DA model developed was robust and reproducibly predictive of pre- and post-HSCT grouping, as demonstrated by X/Y overview values of more than 0.5. Further cross-validation is outside the scope of our study but would require a separate cohort to fully test the model.

Predicted microbiota functions were computationally derived from 88 16S ribosomal RNA (rRNA) sequences spanning pre- and post-HSCT (MG-RAST accession numbers



**FIG 2.** Level 2 PICRUST (phylogenetic investigation of communities by reconstruction of unobserved states) analysis for patients A (A), B (B), and D (C). T (blue)—day of HSCT. N (green)—neutrophil engraftment. Conditioning (yellow): Treo/flu/C1H—treosulfan, fludarabine, Campath. Antibiotics (black): T—teicoplanin; M—meropenem; S—co-trimoxazole; C—ceftazidime; V—vancomycin; A—amikacin; Aug—co-amoxiclav. Clinically significant complications noted (orange).



4634227.3-4634323.3<sup>5</sup>) using PICRUST (phylogenetic investigation of communities by reconstruction of unobserved states), a validated bioinformatics tool.<sup>6</sup> All patients showed fluctuations in relative abundance of predicted microbiota functions (Fig 2). Changes in bacterial functions involved in bacterial motility and membrane transport accounted for most of the fluctuation observed (see Fig E1 this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Only descriptive analysis was undertaken to avoid inferring more generalizable conclusions from a predictive computational modeling technique. As such we did not undertake PLS-DA modeling to demonstrate pre- and post-HSCT groups and further transcriptomic work is required.

Patient A who presented at age 7 months with pneumocystis pneumonia showed more fluctuation in bacterial motility and membrane transport predicted functions (Fig E1, A) than did patients B and D who were under 1 week old at presentation (Fig E1, B and C, E and F). Increases in cell motility functions were associated with the appearance of *Escherichia* species with or without *Klebsiella* species in all patients at pre- and post-HSCT time points, with the exception of day -17 to -4 pre-HSCT for patient A in whom these fluctuations were associated with dominance of *Clostridia* cluster XIVa (Fig E1, A-C). Increases in membrane transporters over HSCT for patient A were associated with appearance of *Enterococcus* species at day -40 and days +44 to +71 and *Clostridia* cluster XIVa from day -17 to -4 (Fig E1, D). Association between fluctuations in functions and specific bacterial taxa would be expected given that functional data are computationally derived from 16S rRNA bacterial taxa data. Some fluctuations predicted bacterial motility and transport functions were associated with time between HSCT and neutrophil engraftment for patient B (Fig 2, B; Fig E1, B and E) and development of fevers and grade II skin GvHD from day +30 in patient D (Fig 2, C; Fig E1, C and F). No other clinical correlations were apparent. These patient-specific changes are difficult to generalize in this pilot study but may represent periods of dysbiosis, a process implicated in gastrointestinal immune-mediated pathologies.

Although UPLC-MS/MS methodology was optimized to detect the maximum number of sample metabolites, this study did not identify specific metabolites. Future work should focus on identification of specific bacterial metabolites with immunoregulatory potential. Butyrate, a short-chain fatty acid produced by colonic bacteria, increases peripheral regulatory T lymphocytes *in vitro* and *in vivo*, through histone deacetylase inhibition (HDACi<sup>7</sup>). Vorinostat, another HDACi, gave low rates of moderate-severe acute GvHD in combination with standard GvHD prophylaxis in a phase I/II clinical trial.<sup>8</sup> Further work is needed to define the role of butyrate, butyrate-producing bacteria, and HDACi in HSCT in the SCID population. Genes involved in flagella assembly were predicted to fluctuate and although testing this prediction through metagenomic sequencing is outside the scope of this study, future work is warranted given the suggested role of flagellin in GvHD prevention in mice.<sup>9</sup>

HSCT disrupts structure and function of the gut microbiota in SCID, which is an important consideration when current treatment involves significant use of antibiotics and chemotherapy with gastrointestinal toxicity.

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## IgE antibodies and response to cow's milk elimination diet in pediatric eosinophilic esophagitis



### To the Editor:

There are 3 lines of evidence supporting cow's milk as a relevant problem food for pediatric patients with eosinophilic esophagitis (EoE). First, studies of the 6-food elimination diet followed by reintroduction of food groups showed that when cow's milk was reintroduced, symptoms or inflammation returned in up to 74% of patients. Second, it was subsequently reported in a retrospective analysis that a significant proportion of patients with EoE (65%) responded to single food elimination of cow's milk.<sup>1</sup> In our own recent prospective study of patients treated with cow's milk elimination, we also found that 65% of the patients had less than 15 eosinophils/hpf after 6 to 8 weeks of treatment.<sup>2</sup> Finally, a striking proportion of pediatric patients with EoE have low levels of serum IgE antibodies to cow's milk.<sup>3</sup> Our objective for the current analysis was to evaluate the relationship between specific

## METHODS

### Patients and samples

Patients and sample acquisition have been previously described.<sup>E1</sup> Briefly, clinical data and longitudinal daily stool samples were collected following informed parental consent. Stool samples were stored at  $-20^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$  before analysis. All 88 samples spanning 1 to 3 months pre- and post-HSCT for 3 patients with SCID who had 16S rRNA gene sequences were subjected to PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states<sup>E2</sup>) computational prediction of microbiota gene functions. Fifty-four samples spanning 1 to 3 months pre- and post-HSCT for 3 patients with SCID were selected for metabolomics analysis using UPLC-MS/MS based on known 16S rRNA gene profiles and occurrence of clinical events.

### Prediction of the bacterial gene functions from 16S rRNA gene sequences

Eighty-eight sequences from our previous study<sup>E1</sup> were obtained from MG-RAST (accession numbers 4634227.3-4634323.3) and analyzed using PICRUSt v1.0<sup>E2</sup> to predict the metabolic function of the bacterial community.

### Metabolomic profiling using UPLC-MS/MS

Water, methanol, and acetonitrile (ACN) were liquid chromatography mass spectrometry (LCMS) grade (Sigma-Aldrich, Dorset, United Kingdom). Metabolites were extracted from 100 mg stool and homogenized in cold 80% methanol by vortexing for 15 minutes at  $4^{\circ}\text{C}$ , centrifuged at 10,000g for 10 minutes at  $4^{\circ}\text{C}$ , and the supernatant was lyophilized in a freeze dryer before storage at  $-80^{\circ}\text{C}$ . Samples were resuspended in 1 mL and diluted a further 1:1 in initial start phase buffer (5% ACN).

Stool metabolite profiling was performed using reverse-phase UPLC-MS/MS. An Accucore C18 column ( $2.6\ \mu\text{m}$ ,  $150 \times 2.1\ \text{mm}$ ) was used at  $40^{\circ}\text{C}$  with

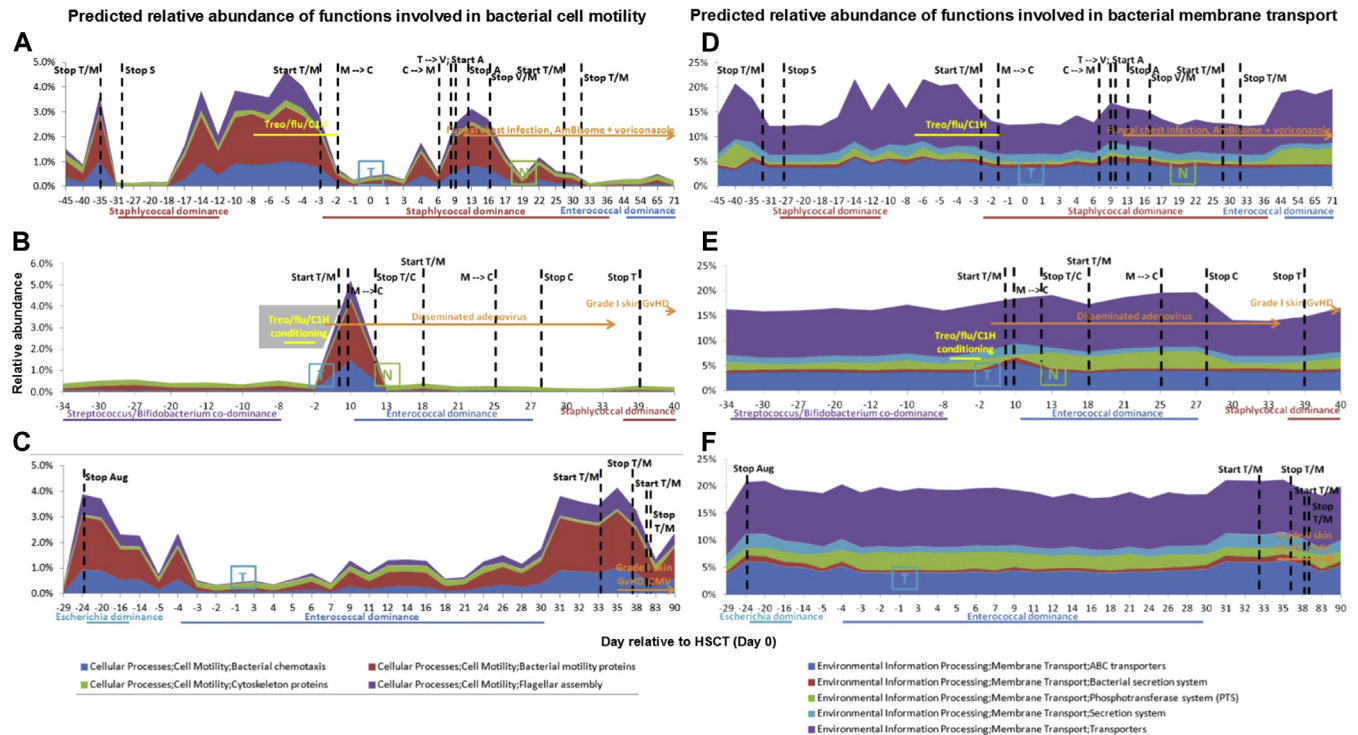
a  $3.0\ \mu\text{L}$  injection and  $300\ \mu\text{L}/\text{min}$  flow rate throughout. A multistep liquid chromatography gradient was used with 5% ACN increasing to 95% ACN over 22 minutes, followed by 8-minute wash and reequilibration. Samples were run randomly in triplicate. A Q-Exactive (Thermo, Wilmington, Mass) was used for the MS, performed using heated electrospray ionization (HESI) with high resolution (70,000) and positive and negative switching. The mass range was set from 100 to 1000 m/z. SIEVE (Version 2.2) was used to process the Thermo RAW files using the recommended thresholds. MS1-based identification of specific metabolites is inaccurate, and significant metabolites did not correspond to in-house standards. Further identification beyond the mass and retention time was not attempted.

### Statistical analysis

Metabolomic profiles from SIEVE (version 2.2) were analyzed by multivariate PLS-DA using SIMCA 13.0 (Umetrics, Stockholm, Sweden<sup>E3</sup>). To check that data were adhering to multivariate normalities, Hotelling's  $T^2$  tolerance limits were calculated and set at 0.95. Coefficient analysis on the PLS-DA data was used to identify metabolites significantly associated with a pre- or post-HSCT metabolite profile at a 95% confidence.

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**FIG E1.** Level 3 PICRUST (phylogenetic investigation of communities by reconstruction of unobserved states) analysis for bacterial cell motility expression (**A-C**) and membrane transport expression (**D-E**) for patients A, B, and D. T (blue)—day of HSCT. N (green)—day of neutrophil engraftment. Conditioning (yellow): Treo/flu/C1H—treosulfan, fludarabine, Campath. Antibiotics (black): T—teicoplanin; M—meropenem; S—co-trimoxazole; C—ceftazidime; V—vancomycin; A—amikacin; Aug—co-amoxiclav. Clinically significant complications noted (orange).

**TABLE E1.** Patient and HSCT details

Patient	Diagnosis	Age at admission (wk)	Age at HSCT (wk)	Conditioning	Donor/ source	Nutrition	Complications
Baby A	X-linked common gamma chain	34	41	Treosulphan, fludarabine, Campath	MUD/cord	NG enteral feeds to day +7; PN day +7 to +45, NG feeds reintroduced gradually from day +16	Pneumocystis jiroveci pneumonia pre-HSCT, slow neutrophil engraftment, fungal chest infection
Baby B	RAG I	<1	6	Treosulphan, fludarabine, Campath	MFD/PBSC	Breast-fed until day −36 then bottle feeds with NG top up feed day +5 to +26 and day +45 onward	Disseminated adenovirus, CVL infection, GvHD skin grade I
Baby D	X-linked common gamma chain	<1	5	Nil	MSD/BMT	Breast-fed throughout except for NG feeds day +39 to +42	GvHD skin grade II, CMV infection

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*BMT*, Bone marrow transplant; *CMV*, cytomegalovirus; *CVL*, central venous line; *MFD*, matched family donor; *MSD*, matched sibling donor; *MUD*, matched unrelated donor; *NG*, nasogastric feeds; *PBSC*, peripheral blood stem cells; *PN*, parenteral nutrition.